Antibiotic Resistance Profiles of Bovine Mastitis-Causing Pathogens Isolated in Ba Vi, Hanoi, Vietnam

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Abstract: The emergence of antibiotic-resistant bacteria has caused significant economic losses in the dairy farm industry due to the decreased effectiveness of treatment in dairy cattle. We conducted antibiotic susceptibility testing against bovine mastitis-causing pathogens isolated from the milk of dairy cattle in the Ba Vi district of Vietnam, a farming region near Hanoi. The isolated bacterial strains examined in this study included 11 strains of *Streptococcus agalactiae* and 7 strains of *Staphylococcus* spp. The strains were tested for resistance to ampicillin, kanamycin, erythromycin, oxytetracycline and cefazolin using the disc diffusion method. All strains were found to be resistant to one or more antibiotics. Of the 18 strains examined, 13 were resistant to multiple antibiotics. In addition, mixed infections involving 2 strains with different antibiotic resistance profiles were confirmed. These results indicate that antibiotic-resistant bacteria are widespread in this region, rendering bovine mastitis more difficult to treat. To ameliorate potential economic losses, it is especially important to use antibiotics correctly.

INTRODUCTION

Bovine mastitis is an economically important disease affecting dairy milk production in Vietnam. As milk consumption in Vietnam has increased over the last 2 decades, domestic production of milk increased markedly following the introduction of highly productive dairy cattle[1]. However, highly lactating dairy cattle require careful nutrition management, as energy imbalances can have significant adverse effects on production[2]. One potential problem affecting milk production is bovine mastitis, an inflammatory disease caused by bacterial infection of the mammary glands[3]. Mastitis-causing pathogens are generally categorized as either environmental or contagious based on the source. The primary contagious pathogens are *Streptococcus agalactiae* and *Staphylococcus aureus*[4]. However, other pathogenic *Staphylococcus* spp. can also cause bovine mastitis[5, 6]. Economic losses associated with bovine mastitis caused by the aforementioned pathogens are typically dueto reduced milk production and treatment costs[3].

Mastitis is commonly treated by the injection and intra-mammary application of ointments of antibiotics such as ampicillin ortetracycline[7]. However, the generation of resistant bacteria selected due to indiscriminate use of antibiotics on farms has reduced the efficacy of treatment in many cases, thus exacerbating economic losses[8]. In addition, although costs can be reduced by initiating treatment as soon as clinical symptoms of mastitis are observed, a subclinical form of mastitis that shows no visible disease signs has been described. Indeed, economic losses due to subclinical mastitis are greater than those due to clinical mastitis. Subclinical mastitis accounts for approximately 80% of mastitis-related lossesdue to reduced milk yield and decreased product quality[9, 10]. Therefore, subclinical mastitis caused by antibiotic-resistant pathogens represents one of the most serious economic threats to the dairy industry.

Previously, we conducted an epidemiologic study of bovine diseases and farm management in the Ba Vi District of Hanoi, Vietnam[11]. In order to identify dairy cattle with subclinical mastitis, milk samples were collected from apparently healthy dairy cows at each farm and then analyzed for mastitis-causing pathogens. A total of 18 isolates of pathogens that cause contagious mastitis were obtained from 153 milk samples. The acquisition of antibiotic resistance by these isolates would further compound the economic losses associated with the disease. Therefore, the aim of the presentstudy was to determinethe antibiotic resistance profiles of mastitis-causing pathogens isolated from dairy cattle in Ba Vi, Vietnam.

MATERIALS AND METHODS

Bacterial strains: A total of 18 strains were examined in this study, all of which were isolated in a previous study[11]. Details regarding these strains were described in the previous report; therefore, only a brief description is given below. Eleven of the 18 strains were *S. agalactiae* and the remaining 7 strains were *Staphylococcus* spp. Four of the *Staphylococcus* spp. strains were confirmed to be *S. aureus* by PCR examination and the 3 remaining strains were classified as Coagulase-Positive Staphylococci (CPS) other than *S. aureus*. These 18 strains were isolated from milk samples obtained from 16 cows. Mixed infections involving *S. agalactiae* and *S. aureus* were confirmed in 2 cows.

Antibiotic susceptibility testing: The antibiotic susceptibility of the strains was examined using thedisc diffusion method according to the procedure recommended by Clinical and Laboratory Standards Institute (CLSI)[12]. First, each strain was cultured overnight on Mueller-Hinton (MH) agar (Becton Dickinson, France) with 5% sheep blood. A total of 4 or 5 colonies were suspended in sterile saline and the turbidity of the suspension was adjusted to that of McFarland 0.5. The bacterial suspension was inoculated onto an MH agar plate and then 5 antibiotic discs were simultaneously placed on the surface using a multi-disc dispenser. The discs contained the following antibiotics: ampicillin (disc content: 10 μg), kanamycin (30 μg), erythromycin (15 μg), oxytetracycline (30 μg) and cefazolin (30 μg). Following incubation at 37°C for 18 h, the diameter of the zone of growth inhibition around each disc was measured using a caliper. The results of antibiotic resistance tests were interpreted according to criteria published by the CLSI. After evaluating the susceptibility of all strains to each antibiotic, the frequency of occurrence of resistant *S. agalactiae* and *Staphylococcus* spp. (*S. aureus* and CPS) was analyzed using statistical software R, version 3.03. The frequency distributions of variables were evaluated using Fisher's exact test and $p<0.05$ were regarded as statistically significant.

RESULTS

All strains were found to be resistant to one or more of the antibiotics tested. Ampicillin exhibited the highest frequency of resistant strains (Table 1). Approximately 77.8% (14/18) of the isolates were ampicillin resistant and the remaining 22.2% (4/18) of ampicillin-susceptible strains were kanamycin resistant. Kanamycin resistance was the second most common among the strains, 66.7% (12/18) of all strains exhibiting resistance to this antibiotic. With regard to the other antibiotics, 50.0% (9/18), 44.4% (8/18) and 5.6% (1/18) of the isolates exhibited resistance to erythromycin, oxytetracycline and cefazolin, respectively.

The frequency of resistance to each antibiotic differed between *S. agalactiae* and *Staphylococcus* spp. There was a significant difference in kanamycin resistance between bacterial species. The rates of kanamycin resistance among *S. agalactiae* and *Staphylococcus* spp. strains were 91% (10/11) and 29% (2/7), respectively. Kanamycin-resistant strains were statistically more common among *S. agalactiae* than *Staphylococcus* spp. ($p = 0.013$). No statistically significant differences were observed for the other antibiotics but a disproportionate trend in the frequency of emergence of resistant strains was observed for some antibiotics. Resistance to oxytetracycline tended to be more common among *S. agalactiae* strains. The proportion of oxytetracycline-resistant *S. agalactiae* strains was 63.6% (7/11) and that of *Staphylococcus* spp. was 14.3% (1/7) ($p = 0.066$). In addition, ampicillin resistance was observed among all isolates of *Staphylococcus* spp. whereas only 63.6% (7/11) of *S. agalactiae* strains were resistant to ampicillin ($p = 0.119$).

Table 1: Antibiotic resistant profiles of mastitis causative isolates and the antibiotic resistance rates

		Antibiotics				
Isolate ID	Species	Ampicillin	Kanamycin	Erythromycin	Oxytetracycline	Cefazolin
1	*S. agalactiae*	Susceptible	Resistance	Susceptible	Susceptible	Susceptible
2	*S. agalactiae*	Susceptible	Resistance	Susceptible	Susceptible	Susceptible
3*	*S. agalactiae*	Resistance	Resistance	Intermediate	Intermediate	Susceptible
4	*S. agalactiae*	Resistance	Resistance	Susceptible	Intermediate	Susceptible
5	*S. agalactiae*	Resistance	Susceptible	Intermediate	Resistance	Intermediate
6	*S. agalactiae*	Susceptible	Resistance	Susceptible	Resistance	Susceptible
7	*S. agalactiae*	Susceptible	Resistance	Resistance	Resistance	Susceptible
8**	*S. agalactiae*	Resistance	Resistance	Resistance	Resistance	Susceptible
9	*S. agalactiae*	Resistance	Resistance	Resistance	Resistance	Susceptible
10	*S. agalactiae*	Resistance	Resistance	Resistance	Resistance	Susceptible
11	*S. agalactiae*	Resistance	Resistance	Resistance	Resistance	Susceptible
12*	*S. aureus*	Resistance	Susceptible	Intermediate	Intermediate	Intermediate
13**	*S. aureus*	Resistance	Intermediate	Susceptible	Susceptible	Intermediate
14	*S. aureus*	Resistance	Susceptible	Susceptible	Susceptible	Susceptible
15	*S. aureus*	Resistance	Susceptible	Resistance	Resistance	Susceptible
16	CPS	Resistance	Susceptible	Resistance	Susceptible	Susceptible
17	CPS	Resistance	Resistance	Resistance	Intermediate	Susceptible
18	CPS	Resistance	Resistance	Resistance	Susceptible	Resistance
Drug resistance rate in all isolates		77.8% (14/18)	66.7% (12/18)	50.0% (9/18)	44.4% (8/18)	5.6% (1/18)
S. agalactiae		63.6% (7/11)	90.9% (10/11)	45.5% (5/11)	63.6% (7/11)	0% (0/18)
S. aureus and CPS		100% (7/7)	28.6% (2/7)	57.1% (4/7)	14.3% (1/7)	14.3% (1/7)
p-value		0.119	0.013	1	0.066	0.389

*ID 3 and 12 were isolated from same milk sample; **ID 8 and 13 were isolated from same milk sample

More than 70% (13/18) of the strains were resistant to multiple antibiotics. Two strains of *S. agalactiae* were resistant to only kanamycin and 3 *S. aureus* strains were resistant to only ampicillin. That is, strains that exhibited resistance to erythromycin, oxytetracycline, or cefazolin were all multi-antibiotic resistant. For example, only one cefazolin-resistant strain of CPS exhibited resistance to four antibiotics: ampicillin, kanamycin, erythromycin and cefazolin. In terms of bacterial species, 81.8% (9/11) of *S. agalactiae* isolates and 57.1% (4/7) of *Staphylococcus* spp. isolates exhibited multi-antibiotic resistance. There was no statistically significant difference in the frequency of acquisition of resistance to multiple antibiotics between *S. agalactiae* and *Staphylococcus* spp. ($p = 0.326$).

DISCUSSION

Two important observations emerged from this study. First, all mastitis-causing strains isolated in cattle in the BaVi district were antibiotic resistant, suggesting the wide spread of resistant bacteria in this region is related to the abuse and/or misuse of antibiotics. Although, cefazolin is still effective for the treatment of mastitis as shown by the antibiotic susceptibility testing results, the use of ampicillin, kanamycin and erythromycin is unlikely to exert the expected therapeutic effect. Ampicillin is frequently used by farmers and veterinarians, as it is the primary therapeutic option for bovine mastitis. However, mastitis poses an even greater economic threat to farmers due to the use of antibiotics that are not effective. Second, the antibiotic resistance profile varied depending on the bacterial species. This could cause serious problems for farmers. Mixed infections involving strains with different antibiotic resistance profiles can severely limit the repertoire of antibiotics available for treatment. Indeed, mixed infections involving both *S. agalactiae* and *S. aureus* were confirmed in 2 cows in our previous study. The results of antibiotic susceptibility testing suggested that *S. agalactiae* develops kanamycin resistance more readily than *Staphylococcus* spp. The bacterial isolates examined in this study were collected from apparently healthy dairy cattle but the history of antimicrobial therapy in these cows was not known. It cannot be denied that kanamycin has been used to treat cattle in the past but it is difficult to assume that kanamycin was administrated more frequently in *S. agalactiae* infected cows than *S. aureus* infected cows because infection with each of these bacteria produce the same symptoms in dairy cows. Mutations in the ribosomal 30S subunit, the target site for kanamycin, are considered to be the principal mechanism leading to kanamycin resistance in both *S. agalactiae* and *Staphylococcus* spp.[13]. The observed differences in resistance frequency could be due to another mechanism, such as a decrease in the permeability of the cell membrane to the antibiotic. Further research is required to clarify this discrepancy.

CONCLUSION

All causative pathogens of bovine mastitis in Ba Vi, Vietnam, are likely antibiotic resistant. Importantly, the present study found that most of these pathogens are resistant to multiple antibiotics. The high frequency of antibiotic-resistant bacteria and mixed infections

involving strains with different antibiotic resistance profiles make mastitis difficult to treat, thus leading to greater economic losses for farmers. We hope that the results of this study will lead to a re-examination of the use of antibiotics.

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